

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE
in its capacity as elected Office

Date of mailing: 22 March 2001 (22.03.01)	
International application No.: PCT/US00/25186	Applicant's or agent's file reference: 03940005TA
International filing date: 15 September 2000 (15.09.00)	Priority date: 16 September 1999 (16.09.99)
Applicant: TOWNSEND, Craig, A. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International preliminary Examining Authority on:
21 December 2000 (21.12.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer: J. Zahra Telephone No.: (41-22) 338.83.38
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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: MICHAEL E. WHITHAM
MCGUIREWOODS, LLP
1750 TYSONS BLVD
SUITE 1800
MCLEAN, VA 22102

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 08940005TA	Date of Mailing (day/month/year) 16 JAN 2001
International application No. PCT/US00/25188	International filing date (day/month/year) 15 SEPTEMBER 2000
Applicant THE JOHNS HOPKINS UNIVERSITY	

1. ☐ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
 The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
 34, chemin des Colombettes
 1211 Geneva 20, Switzerland
 Facsimile No.: (+1-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 bis 1 and 90 bis 3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PETER PARAS JR. Telephone No. (703) 308-0196
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PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 03940005TA	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US00/25186	International filing date (day/month/year) 15 SEPTEMBER 2000	(Earliest) Priority Date (day/month/year) 16 SEPTEMBER 1999
Applicant THE JOHNS HOPKINS UNIVERSITY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
 - ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 25.1(b)).
- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
 - ☐ contained in the international application in written form.
 - ☐ filed together with the international application in computer readable form.
 - ☐ furnished subsequently to this Authority in written form.
 - ☐ furnished subsequently to this Authority in computer readable form.
 - ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the
 - ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
- 2. ☐ **Certain claims were found unsearchable** (See Box I).
- 3. ☐ **Unity of invention is lacking** (See Box II).
- 4. With regard to the **title**,
 - ☒ the text is approved as submitted by the applicant.
 - ☐ the text has been established by this Authority to read as follows:
- 5. With regard to the **abstract**,
 - ☒ the text is approved as submitted by the applicant.
 - ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
- 6. The figure of the **drawings** to be published with the abstract is Figure No. ____

- ☐ as suggested by the applicant.
 - ☐ because the applicant failed to suggest a figure.
 - ☐ because this figure better characterizes the invention.

☒ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/25186

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : 536/23.1; 435/ 252.3, 320.1, 326, 471

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/ 252.3, 320.1, 326, 471

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,869,299 A (BAGGALEY et al) 09 February 1999 (09.02.99), see entire document.	1-30
Y	Database Caplus on STN, No. 583939, KHALEELI, N. et al. 'Origin of the beta-lactam carbons in clavulanic acid from an unusual thiamine pyrophosphate-mediated reaction,' abstract, J. Am. Chem. Soc, 1999, 121(39), 9223-9224.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 DECEMBER 2000

Date of mailing of the international search report

16 JAN 2001

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A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

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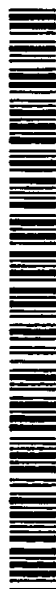
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- Before the expiration of the time limit for amending the
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For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: IMPROVEMENT OF CLAVULANIC ACID PRODUCTION

(57) Abstract: The invention methods for the enhancement of clavulanic acid production. In particular, the invention provides a method for increasing the production of clavulanic acid by: gene dosage with *orf2* from the clavulanic acid biosynthetic pathway in *Streptomyces clavuligerus*; and by manipulation of fermentation conditions, especially the concentration of DG3P, a substrate of N2(carboxyethyl)arginine synthase, the protein encoded by *orf2*. A method for preparing N2(carboxyethyl)arginine synthase is also provided, as is an assay for identifying its substrates.



WO 01/19959 A1

IMPROVEMENT OF CLAVULANIC ACID PRODUCTION

This invention was made using funds from grants from the National Institutes of Health having grant number AI 14937. The government may have certain rights in this invention.

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention generally relates to the enhancement of clavulanic acid production. In particular, the invention provides a method for increasing the production of clavulanic acid by: gene dosage with *orf2* from the clavulanic acid biosynthetic pathway in *Streptomyces clavuligerus*; and by manipulation of fermentation conditions, especially the concentration of D-G3P, a substrate of N2(carboxyethyl)arginine synthetase, the protein encoded by *orf2*. A method for preparing N2(carboxyethyl)arginine synthetase is also provided, as is an assay for identifying its substrates.

Background of the Invention

The β -lactams such as penicillin, cephalosporin and cephamycin were the first useful class of antibiotics discovered and are still in clinical use to combat infections. However, the extensive use of β -lactams has reduced their effectiveness due to the emergence of resistance among invading pathogens. There is therefore an increasing urgency to develop strategies aimed at overcoming acquired resistance.

Resistance to the antibiotics penicillin and cephalosporin is conferred upon bacteria via the β -lactamase enzymes. β -lactamases catalyze the hydrolysis of the antibiotics, rendering them inactive. One of the best examples of strategies aimed at overcoming acquired resistance is the development of β -lactamase inhibitors, exemplified by commercial products such as AUGMENTIN® and TIMENTIN®. These products are combinations of clavulanic acid, (a potent, naturally occurring β -lactamase inhibitor that is produced by the bacterium *Streptomyces clavuligerus*) together with other β -lactam antibiotics. As a result of this widespread clinical application, clavulanic acid production is currently valued in excess of a billion dollars.

With the advent of recombinant DNA technology and protein engineering, there has been increasing interest in manipulating biochemical pathways, especially those which produce commercially viable compounds. This may be accomplished, for example, by amplifying the genes encoding the rate-limiting enzymes of the pathway, or by modifying enzyme specificity. To effectively enhance productivity by genetic manipulation, identification of the controlling steps in the biosynthetic pathway is essential. Frequently an enzyme involved in the conversion of primary metabolites to the first dedicated intermediate in the biosynthetic pathway for the secondary metabolite has been identified as the rate-determining enzyme. Increasing the gene dosage of the rate-determining enzyme may result in the overproduction of flux-limiting enzyme and lead to increased production of the final product. This is the case, for example, in *Cephalosporium acremonium* and *Streptomyces clavuligerus* where the non-ribosomal condensation of the ACV tripeptide from α -aminoadipic acid, valine and cysteine is the rate-determining step in the penicillin pathway (Malmberg, L.-H., Hu, W.-S. and Sherman, D.H. 1993, *J. Bacteriol.* 175:6916-6924). However, for this approach to be viable, identification of all enzymes involved in the pathway is required, as well as a detailed kinetic analyses for each reaction step.

Clavulanic acid is produced by fermentation processes employing the bacterium *Streptomyces clavuligerus*. While the gene cluster responsible for clavulanic acid biosynthesis in this organism has been identified (Li, R.-F., Khaleeli, N. and Townsend, C.A. 2000, *J. Bacteriol.* 182:4087-4095), the complete biosynthetic pathway has not been sufficiently well-characterized to identify the rate-limiting reactions of the pathway. Therefore, it is currently not possible to rationally design recombinant DNA approaches to increasing clavulanic acid production. It would be highly desirable to elucidate the biosynthetic pathway of clavulanic acid and to utilize the information in order to enhance production of this clinically valuable compound by genetic manipulation.

SUMMARY OF THE INVENTION

The invention relates to the discovery that D-glyceraldehyde-3-phosphate is a primary metabolic precursor of clavulanic acid in an unusual thiamin pyrophosphate (TPP)-mediated reaction carried out by N2(carboxyethyl)arginine synthase. The enzyme is encoded by *orf2* in the producing organism, *Streptomyces clavuligerus*. Improvement of clavulanic acid production can be achieved by gene dosage and by the design/manipulation of fermentation conditions to attain favorable levels of D-G3P or L-arginine for synthesis. In particular, the invention provides a method for increasing the production of clavulanic acid by amplification of the *orf2* gene in an appropriate host, for example *S. clavuligerus* bearing

the intact pathway, and a rationale for improvements to fermentation conditions. The invention also provides a method for preparing the enzyme N²-(2-carboxyethyl)arginine synthase; and an assay for detecting potential substrates of N²-(2-carboxyethyl)arginine synthase.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of plasmid pKC1139/pro-*orf2*-ter.

Figure 2. Fermentation of *S. clavuligerus* strains: *orf2* gene dosage study employing native promoter and replicating vector. ♦ = WT/pKC1139/pro-*orf2*-ter (in SA+ medium); ■ = WT (in SA+ medium).

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Figure 3. Construction of integrative plasmid pSET152/pro-*orf2*.

Figure 4. Southern hybridization analysis of chromosomal DNA isolated from recombinant strains using *orf2* probe. Lane 1. WT/pSET152-EcoRI; Lane 2. WT/pSET152/*ermE*(XbaI)-*orf2*-EcoRI; Lane 3. WT/pSET152/pro-*orf2*-EcoRI/HindIII; Lane 4. WT/pSET152-EcoRI/HindIII; Lane 5. WT/pSET152/*ermE*(HindIII)-*orf2*-EcoRI/HindIII; Lane 6. 2-2-23/pSET152/*ermE*(HindIII)-*orf2*-EcoRI/HindIII.

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Figure 5. Fermentation of *S. clavuligerus* strains: *orf2* gene dosage study employing native promoter and integration vector. ■ = WT/pSET152/pro-*orf2* (in SA+ medium); ♦ = WT (in SA+ medium).

Figure 6. Construction of integration vector pSET152/*ermE*(XbaI)-*orf2*.

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Figure 7. Construction of integration vector pSET152/*ermE*(HindIII)-*orf2*.

Figure 8. Fermentation of *S. clavuligerus* strains: *orf2* gene dosage study employing *ermE* promoter and integration vector. ♦ = WT; ■ = WT/pSET152*ermE* (XbaI)-*orf2*; ● = WT/pSET152*ermE* (HindIII)-*orf2*.

**DETAILED DESCRIPTION OF THE PREFERRED
EMBODIMENTS OF THE INVENTION**

As illustrated in the Examples below, we have discovered that the enzyme encoded by *orf2* of the clavulanic acid gene cluster catalyzes the first biosynthetic reaction in the clavulanic acid pathway (Scheme 1). It mediates the condensation of two primary metabolites, D-glyceraldehyde-3-phosphate (D-G3P) and L-arginine, to give the first intermediate N²-(2-carboxyethyl)-L-arginine (CEA). The level of Orf2 therefore controls the uptake of both precursors from primary metabolism to clavulanic acid production.

Although the genetic sequence of *orf2* and the deduced amino acid sequence have been previously identified by Jensen et al. (Canadian Patent 2,108,113) the function of Orf2 that is suggested by Jensen et al. (acetohydroxyacid synthase-like activity, i.e. participation in the biosynthesis of branched chain amino acids) is incorrect. As shown in Example 1 below, the present disclosure provides the correct description of the enzymatic activity of the *orf2* gene product, N²-(2-carboxyethyl)arginine synthase, including elucidation of its substrates D-G3P and L-arginine.

Based on this discovery, we investigated whether an increase in *orf2* gene expression would increase the flux of precursors from the primary metabolite pool into the clavulanic acid pathway and improve clavulanic acid yield. It is known that the introduction and expression of homologous or heterologous gene(s) involved in antibiotic biosynthesis in a producer strain can be a way to enhance productivity, to affect percent composition of a desired final product, or to synthesize hybrid antibiotics. For example, a 40% increase in penicillin production was obtained by transforming *P. chrysogenum* with extra copies of a DNA fragment containing the genes involved in the last two steps of the pathway (Veenstra, A.E., P. van Solingen, R.A. L. Bovenberg and L.M.H. van der Voort, 1991. *J Biotechnol.* 17:81-90.). Accordingly, as illustrated in the Examples below, we cloned *orf2* and its upstream regulatory sequence into both replicative and integrative vectors. The results showed that clavulanic acid production levels in recombinant strains increased by 53% and 68%, respectively. We also placed *orf2* under the transcriptional control of the constitutive and strong *ermE** promoter. The results showed that the recombinant strains produce 34% to

68% more clavulanic acid than the wild-type strain. Further, Southern hybridization showed that all recombinant strains constructed contained the additional copy of *orf2* integrated into the chromosome.

The present invention provides a method for increasing the production of clavulanic acid in *Streptomyces clavuligerus*. The method involves increasing the production of the enzyme N²-(2-carboxyethyl)arginine synthase in the bacterium. In one embodiment of the present invention, the increase is effected by providing the bacterium with additional copies of the DNA that encodes the enzyme. By "additional copies of the DNA", we mean copies of the DNA that are introduced into the bacterium via genetic engineering techniques.

By "DNA that encodes the enzyme" we mean any sequence of nucleotides that encodes a functional N²-(2-carboxyethyl)arginine synthase enzyme. Those of skill in the art will recognize that this may include the "native" enzyme (including any elements such as native control elements e.g. promoters, ribosome binding sites, terminators and the like) or many variations of the "native" enzyme, including but not limited to forms of the enzyme with conservative amino acid substitutions, non-conservative amino acid substitutions, insertions, deletions, truncations, fusions, and the like. The enzyme may be genetically engineered, for example, in order to maximize such parameters as substrate binding efficacy, rate of catalysis, stability, molecular weight, or to produce chimeric proteins, or to carry out directed evolution or gene shuffling, and the like. DNA encoding any suitable form of N²-(2-carboxyethyl)arginine synthase may be used in the practice of the present invention, so long as the resulting form of the enzyme carries out the enzymatic reaction of the condensation of two primary metabolites, D-glyceraldehyde-3-phosphate (D-G3P) and L-arginine to give the first intermediate in the clavulanic biosynthetic pathway, N²-(2-carboxyethyl)-L-arginine (CEA). The present invention is intended to encompass all such forms of the enzyme, as well as the DNA sequences which encode them.

The DNA sequence itself may be of any suitable sequence that encodes a functional form of the N²-(2-carboxyethyl)arginine synthase enzyme. The DNA sequence may be modified in any of a variety of appropriate ways, including but not limited to: the introduction of restriction enzyme sites, manipulation of the sequence to facilitate cloning or handling of the DNA, to create chimeric forms of the protein, to effect alterations in the

amino acid sequence of the encoded enzyme, to increase or decrease stability of the DNA itself, or of the encoded enzyme. Such modifications may include various appropriate chemical modifications, the introduction of various control elements and manipulation of their location (e.g. promoters, ribosome binding sites, terminators), and the like. Any suitable form of DNA encoding a functional form of the N²-(2-carboxyethyl)arginine synthase enzyme may be used in the practice of the present invention.

The production of N²-(2-carboxyethyl)arginine synthase in *Streptomyces clavuligerus* is a controlled event. It may be controlled by positive or negative regulators of transcription, as well as other factors in the fermentation medium. Modulation of production may thus be controlled, for example, by the deletion of a copy of a negative regulator (e.g. a transcription factor) or by the insertion of additional copies of a positive regulator (transcription factor), or by modifying the stress (fermentation) conditions in a manner that alters the activity or production of such factors. For example, a transcription factor that upregulates the transcription of *orf2* may be added to the fermentation medium.

The form of the N²-(2-carboxyethyl)arginine synthase enzyme utilized in the methods of the present invention may be the native form of the enzyme, or may be any of various other modified forms of the enzyme. Examples of such modifications include but are not limited to post-translational modifications carried out within a host organism (e.g. acylation, glycosylation, phosphorylation, and the like), or *in vitro* modifications (e.g. chemical modifications, proteolytic modifications, labeling, attachment to a substrate, and the like). Any form of the enzyme that is competent to carry out the condensation of D-glyceraldehyde-3-phosphate (D-G3P) and L-arginine, to yield N²-(2-carboxyethyl)-L-arginine may be used in the practice of the methods of the present invention. The enzyme may also be fused with another protein to generate a chimeric form of the enzyme.

In one embodiment of the invention, the DNA is provided to the bacterium by the introduction of a plasmid encoding the N²-(2-carboxyethyl)arginine synthase gene. In a preferred embodiment of the present invention, the plasmid is the replicating plasmid pKC1139/pro-*orf2*-ter. However, those of skill in the art will recognize that many other plasmid vectors may also be utilized in the successful practice of the present invention. For example, plasmids such as pIJ680, pIJ702, pWHM1109, and pKC1218 may also be used.

Any suitable plasmid that provides within the bacterium a DNA sequence encoding an appropriate form of N²-(2-carboxyethyl)arginine synthase may be utilized in the practice of the present invention.

In one embodiment of the present invention, the DNA that encodes the N²-(2-carboxyethyl)arginine synthase gene is integrated into the host genome. The invention thus also provides a host cell in which the *orf2* gene is stably integrated. In one embodiment of the present invention, the integration is carried out utilizing an integrative vector which may, for example, be a site-specific integrative vector. In preferred embodiments, the site-specific integrative vectors are pSET152/pro-*orf2*, pSET152/*ermE*(XbaI)-*orf2* or pSET152/*ermE*(HindIII)-*orf2*. However, those of skill in the art will recognize that other integrative vectors may also be used in the practice of the present invention, for example pOJ436, pOJ444 and pGM9. Any suitable integrative vector that results in the stable integration of a DNA sequence encoding an appropriate form of N²-(2-carboxyethyl)arginine synthase may be utilized in the practice of the present invention.

In one embodiment of the present invention, the DNA sequences that encode N²-(2-carboxyethyl)arginine synthase may include a promoter. The promoter may be the native promoter, or a promoter that has been genetically engineered into the DNA. The promoter may be a constitutive promoter and may be a promoter that is recognized by those of skill in the art as a strong promoter. In a preferred embodiment of the present invention, the promoter is the *ermE** promoter. However, those of skill in the art will recognize that many suitable promoters exist which may be used in the practice of the present invention, for example, *PtipA*, *aph* and *xyl*. Any suitable promoter that results in appropriate expression of DNA encoding N²-(2-carboxyethyl)arginine synthase may be utilized in the present invention. In addition, the sequence of any given promoter may also be altered, for example, to provide ease of genetic manipulation, or to modulate the relative strength of the promoter.

In a preferred embodiment of the present invention, the bacterium which is utilized for enhanced production of clavulanic acid is *Streptomyces clavuligerus*. However, those of skill in the art will recognize that other host organisms may also be utilized in the practice of the present invention. For example, other *Streptomyces* such as *S. lividans*, *S. coelicolor*, *S. jumonjinensis* (e.g. ATCC 29864), *S. lipmanii*, *S. katsurahamanus* (e.g. strain T272), *S.*

parvulus, *S. griseofulvus*, and *S. antibioticus*. In addition, host organisms may not be limited to bacterial hosts but may include other expression hosts such as yeast, plant cells, or cultured cells. Any host capable of carrying out the biosynthesis of clavulanic acid may be utilized in the practice of the present invention.

5 The invention further provides a method to increase clavulanic acid production by effecting alterations in the bacterial growth conditions, e.g. precursor concentration, fermentation conditions, additives such as dihydroxyacetone, glycerol, inositol and glucuronate, etc. Those of skill in the art will recognize that many such parameters can be altered and all such variations are intended to be within the scope of the present invention.

10 For example, the concentration of the substrates utilized by the enzyme N²-(2-carboxyethyl)arginine synthase (D-glyceraldehyde-3-phosphate and L-arginine) may be increased. This may be accomplished in any of a variety of ways, including but not limited to: the addition of those substances or precursors of those substances to the growth medium, either directly, or by the introduction of or genetic manipulation of genes which, either

15 directly or indirectly, enhance their production; and effecting a decrease in the breakdown of the substrates (e.g. by reducing the activity of glyceraldehyde dehydrogenase), or modulating the diversion of the substrates into other pathways, for example by modulating other enzymatic pathways in which they participate. In addition, the availability of TPP may be increased. Other fermentation conditions such as temperature, ionic strength, nutrient levels,

20 and the like may also be altered. Any fermentation parameter which has the effect of increasing the concentration of, or increasing the activity of the enzyme N²-(2-carboxyethyl)arginine synthase, may be altered in the practice of the method of the present invention.

25 The present invention also provides a method for preparing the enzyme N²-(2-carboxyethyl)arginine synthase. In general, the method comprises the steps of culturing and harvesting a host cell which synthesizes the enzyme, obtaining an appropriate enzyme-containing fraction from the host (for example, the supernatant after a centrifugation step), subjecting the fraction to ammonium sulfate precipitation, resuspending the precipitated protein pellet, and chromatographing the resuspended protein on an affinity column. In a

30 preferred embodiment of the present invention, the ammonium sulfate precipitation utilized

is a 30% precipitation. In a preferred embodiment of the present invention, the affinity column is an L-arginine agarose affinity column.

In a preferred embodiment of the present invention, the cells were lysed in 50 mM N-[2-acetamido]-2-iminodiacetic acid (ADA) pH 6.0, 5 mM MgCl₂, 0.5 mM thiamine pyrophosphate, 1 mM DTT, 2 mM EDTA and 12.5 ug/mL Trypsin Chymotrypsin Inhibitor. This buffer, ADA pH 6.0, was chosen so as to approach the theoretical pI (5.1) of the protein without adversely affecting the activity in preparation for an isoelectric precipitation (the salting out of proteins at pHs near their theoretical pIs). Cellular debris was removed by centrifugation and finely ground ammonium sulfate was added to the cell free extract to a final concentration of 30%. The protein solution was incubated on ice for 15 min with stirring. Centrifugation at 13,000 x g yielded a protein pellet which was resuspended in 1 mL buffer [25% glycerol, 100 mM TrisHCl pH 8.0, 5 mM MgCl₂, 12.5 ug/mL Trypsin Chymotrypsin inhibitor, 0.5 mM TPP and 1 mM DTT with rotary shaking on ice. Two distinctive features of this precipitation are the pH of the precipitation and resuspension buffers and their glycerol contents.

In a preferred embodiment of the preparation method, the host cell is *Escherichia coli*. However, those of skill in the art will recognize that any host cell capable of producing N²-(2-carboxyethyl)arginine synthase may be utilized in the method of the present invention.

The present invention also provides an assay for the identification of potential substrates of N²-(2-carboxyethyl)arginine synthase. Potential substrates include those which result in novel products (i.e. products which are not CEA) which could undergo biotransformation novel forms of antibiotics. The substrates may be naturally occurring or synthetic. For example, by modifying either the substrate L-arginine or D-G3P at atoms which are not directly involved in the enzymatic reaction, it is possible to retain the ability of those substrates to be acted upon by the enzyme, and produce a condensation product that is not CEA but which includes the modification. Examples include the attachment of a moiety of interest to the side chain of arginine, such that the moiety is retained in the reaction product. The resulting condensation product may go on to be acted on by other enzymes and thus produce other novel substances, or the product may be desirable in its own right.

The assay is carried out by incubating a potential substrate with the enzyme in the

presence of TPP and one known substrate and detecting the presence or absence of a condensation product that is produced. If the result is positive (i.e. if a condensation product is detected) then the putative substrate may be deemed a substrate of the enzyme. If the potential substrate is intended to replace or mimic arginine, then the known substrate may be D-G3P. Conversely, if the potential substrate is intended to replace or mimic D-G3P, then the known substrate may be arginine. In a preferred embodiment of the present invention, the known substrate may be labeled, for example by radiolabeling, (e.g. [U-¹⁴C]Arginine. The label would then be incorporated into the product so that the product is readily detectable. However, those of skill in the art will recognize that many ways of designing such as assay exist. For example, the product may be detected by utilizing other detectable labels on the known substrate which would be incorporated into the product during the reaction, or monitoring the production of the product by some other method (e.g. HPLC). All such variations are intended to be encompassed by the assay of the present invention.

Further, the process described in the assay procedure may also be utilized in order to create novel condensation products. According to this facet of the invention, the enzyme may be utilized to condense any substances which are capable of acting as substrates for the enzyme in order to produce condensation products.

The present invention also encompasses a method for increasing the production of N²-(2-carboxyethyl)arginine in a host cell by enhancing a rate of condensation of the substrates L-arginine and D-G3P. The enhancement may be effected by the enzyme N²-(2-carboxyethyl)arginine synthase, for example by increasing the copy number of the synthase in the host cell.

The following Examples are included by way of illustration but should not be interpreted to limit the invention in any way.

EXAMPLES

EXAMPLE 1. Origin of the β -lactam Carbons in Clavulanic Acid from an Unusual Thiamine Pyrophosphate-Mediated Reaction

The primary metabolic precursors of clavulanic acid are known to be arginine^{1,2} and a C3-intermediate thought to be derived from glycolysis.³ Identification of the latter has defied assiduous investigation over many years.^{4,5} The first gene of the clavulanic acid gene cluster in *Streptomyces clavuligerus* encodes a thiamine pyrophosphate (TPP)-dependent enzyme that carries out the unprecedented condensation of L-arginine with D-glyceraldehyde-3-phosphate (2 in Scheme 2) to give N²-(2-carboxyethyl)arginine (3 in Scheme 1, CEA), the first dedicated intermediate in clavulanic acid biosynthesis.⁶

Detailed isotopic labeling experiments have placed strict constraints on the mechanism of the coupling reaction that links the C₃- and C₅-building blocks drawn from primary metabolism to initiate clavulanic acid biosynthesis. It is known that H-2 of both glycerol (4 in Scheme 2, H_E),^{4,7} and glyceric acid (5 in Scheme 1, H_F)^{5,8} are lost on incorporation into clavulanic acid. Of the four remaining glycerol hydrogens (4 in Scheme 2, H_{A-D}), only one is retained in 1.⁴ The identity of this single hydrogen (H_B) was determined in a stereochemical experiment in which only the *pro*-(*R*) arm of glycerol was radiolabeled specifically at one or the other diastereotopic methylene locus (4, H_A or H_B).⁹ This telling result implied that, since stereochemical information is retained through the intermediates of glycolysis as far as phosphoenol pyruvate (PEP), the biosynthetic pathway must proceed in such a way to transmit this information to clavulanic acid (1). The suggested intermediacy of lactate⁶ or pyruvate,^{8,10} therefore, can be excluded; that is, isotopic labels which are diastereotopic in 4 and 5 become achiral in a methyl group and, consequently, lose their ability to transfer label stereospecifically to clavulanic acid.

Two further observations made it possible to establish the stereochemical course of N—C bond formation in the construction of CEA (3). First, it could be shown that both H_A and H_B in glycerol (4) are completely retained in the formation of proclavaminic acid (6 in Scheme 2).¹¹ Second, incubation of 5 stereospecifically labeled at C-4' (5, H_A or H_B) with clavamate synthase demonstrated that the oxidative cyclization/desaturation to clavaminic

acid (7 in Scheme 2) occurred with clean stereochemical retention. Knowing H_B survives the striking ring inversion to clavulanic acid (1), a complete stereochemical correlation could now be deduced as outlined in Scheme 2. It may be concluded that the C—O bond in 4 and 5 is replaced by the N—C bond in CEA (3) with overall retention of configuration. In sum, these results limit the possible precursor from primary metabolism to a C₃-carbohydrate likely lying between glycerol and PEP whose hydroxymethylene oxidation state is maintained throughout stereospecific CEA formation.

Identification of the 3-carbon unit became possible with the recent discovery of a new biosynthetic enzyme which cyclizes CEA (3) to the β-lactam ring contained in proclavaminic acid (6 in Scheme 2). This ATP/Mg⁺⁺-dependent protein catalyzes a previously unknown reaction type, a β-lactam synthetase, and is encoded by the second gene in the clavulanic acid biosynthetic cluster.^{13, 14} The first gene of the cluster lies directly upstream and gives rise to a protein of translated molecular mass 60, 907 Da showing sequence identities as high as 29% to acetolactate synthases from several sources, and to a lesser extent to pyruvate oxidases. The potential relation of a thiamine pyrophosphate-dependent enzyme such as these to any step in clavulanic acid biosynthesis was not obvious.

To examine the biosynthetic role of its encoded gene, *orf2* was cloned into pET24a (Novagen) and used to transform *E. coli* B834(DE3). As a control, this host was also transformed with the vector alone. The recombinant clones were separately inoculated into LB medium and, once growth had reached A₆₀₀ = 0.7, they were transferred to sterile flasks and induced with IPTG. After 3 h, 1 mM [U-¹⁴C]arginine (50 μCi/mmol) was added and incubation was continued for an additional 21 h at 28 °C. The cells were harvested by centrifugation and the supernatants were analyzed by HPLC after microfiltration [Spherex 18 5μ ODS(4) (Phenomenex), 50 mM ammonium bicarbonate as eluant]. The appearance of radioactivity in the chromatograms was monitored by scintillation counting. Significant radioisotope was detected in samples with a retention time coincident with CEA in the sample from the recombinant bearing *orf2*, but not the control culture. This finding implied that the over-produced protein encoded by the first gene of the biosynthetic cluster catalyzed the condensation of L-arginine with some primary metabolite available in *E. coli* to synthesize CEA (3), that is, the elusive C₃-unit itself.

Preliminary identification of the precursor of the C₃-unit was sought in an *in vitro* experiment. A cell-free extract (CFE) was prepared from frozen cell paste of the recombinant strain according to the method of Busby et al.¹⁵ [U-¹⁴C]Arginine was incubated in the presence of various potential C₃-intermediates (30 mM), TPP (1.5 mM) and the CFE in Tris buffer. The glycolytic intermediates examined were several phosphoglyceric acids (PGA, Table 1), D,L-glyceraldehyde-3-phosphate (D,L-G3P), dihydroxyacetone phosphate (DHAP), phosphoenolphosphate (PEP), D,L-glyceraldehyde (D,L-GA) and pyruvic acid (PA). After 3 h reaction at room temperature, the protein was removed by membrane filtration (Ultrafree 5000, Millipore) and the samples were analyzed by HPLC and scintillation counting as before. Despite our fear that extensive equilibration among the intermediates of glycolysis would cloud the outcome, the results of this experiment were clear, if unexpected. Although low levels of radioactivity appeared in CEA in every trial, the most efficient production of this first biosynthetic intermediate was observed with DHAP (Table 1).

As a first step toward purifying CEA synthase, it was discovered that a fortuitously efficient precipitation of the over-produced enzyme could be carried out with 30% ammonium sulfate. The protein pellet was resuspended and dialyzed¹⁶ to give substantially pure CEA synthase (>95%) when examined by SDS-PAGE. Rescreening of the glycolytic intermediates with the partially purified enzyme gave reduced background counts in CEA and appeared to confirm the identity of DHAP as the precursor of the β -lactam carbons of clavulanic acid from primary metabolism (Table 1). A large-scale incubation of DHAP and L-arginine provided further evidence of this remarkable reaction yielding a single product whose chromatographic behavior and ¹H-NMR spectrum were identical to an authentic specimen of CEA (3).¹³

However, re-evaluation of D,L-G3P at higher concentration (60 mM) gave a greater incorporation in to CEA (3) (Table 1). Suspecting that one or the other enantiomer of G3P might be inhibitory, D-G3P was generated and found to give a conversion to CEA comparable to that of DHAP. It now appeared, therefore, that both DHAP and D-G3P could serve as substrates for the enzyme in the synthesis of CEA.

Triosephosphate isomerase (TIM) mediates the isomerization of DHAP and G3P and

is notorious for its exceptionally high catalytic activity.¹⁷ Even a slight contamination by this enzyme could be responsible for the DHAP/D-G3P interconversion apparently carried out by CEA synthase. To examine this possibility, the substantially pure solubilized 30% ammonium sulfate pellet was loaded onto an L-arginine-agarose affinity column (Sigma) and eluted with a gradient of NaCl to give a highly purified sample of CEA synthase as judged by SDS-PAGE. The conversion of DHAP to D-G3P in a standard TIM assay¹⁸ carried out in presence of the affinity-purified synthase, but in the absence of L-arginine, was significantly less efficient (<0.1%) than that from the 30% ammonium sulfate pellet (*ca.* 20%). Importantly, while the specific activities of the time-dependent transformation of D-G3P + [¹⁴C]-L-arginine to CEA (**3**) in the presence of TPP increased as the purification of the protein advanced, the conversion of DHAP, while initially high in the CFE, fell successively to background when assayed with the affinity purified enzyme. Another round of purification through the affinity step gave homogeneous protein by SDS-PAGE and essentially unchanged activities for these two substrates indicating the trace TIM activity had been removed.

Thiamine pyrophosphate is absolutely required for enzyme activity, in keeping with the translated signature motif noted in Orf2. This is an unusual transformation for this cofactor more commonly associated with C–C bond breaking and bond-forming reactions as, for example, transketolases or the decarboxylation of α -ketoacids. CEA synthase mediates an internal redox reaction and a β -elimination/addition leading to N–C bond formation in the synthesis of **3**. This is a pleasingly adroit process in which the carboxyethyl of the product **3** required for β -lactam formation is generated by the capture of a glycolytic intermediate having the equivalent oxidation state.

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Example 2. Enhancement of clavulanic acid production by amplification of the *orf2* gene cloned into a replicating plasmid:

There is a 495-bp intergenic region between *orf1* and *orf2*. *Orf1* is transcribed divergently to *orf2*, so this region must contain the regulatory sequence that controls the expression of *orf2* and other downstream biosynthetic genes. To ensure that *orf2* cloned on a plasmid will be regulated the same way as the wild-type chromosomal copy of the DNA, *orf2* and its upstream region (2216 bp) were amplified by PCR. A 400-bp DNA fragment containing a terminator sequence that was originally from *fd* virus was excised from *Streptomyces* plasmid pIJ486 (18) and inserted into the *EcoRV*-*Bam*HI digested and T₄ DNA polymerase treated bifunctional vector pKC1139. The *orf2* PCR product was inserted into the *Hind*III site to give rise to the final recombinant plasmid (Figure 1). This plasmid was transformed into wild-type *S. clavuligerus* by protoplast transformation (19). A standard fermentation in regular SA medium (13) containing 1% arginine and 1% glycerol (SA+) was carried out in shake flasks. Samples were taken at different time points during the fermentation and standard imidazole assay was performed (6) (16). Cell densities measured at 600 nm (OD₆₀₀) showed that all cultures grew at similar rates. Figure 2 shows the results from the imidazole assays. *S. clavuligerus* containing pKC1139/*pro-orf2-ter*. produced about 55% more clavulanic acid than wild-type *S. clavuligerus*. Thus, this genetic modification provides a viable means for increasing production of clavulanic acid.

Example 3. Improvement of clavulanic acid yield by using a site-specific integrative vector.

Because plasmids often cause pleiotropic effects on antibiotic production and on the developmental life cycle of producing strains, in some cases the presence of replicating plasmids can considerably reduce antibiotic production (3). To investigate whether this is the case for an *orf2* gene dosage study in *S. clavuligerus*, we decided to use the integration method to insert a additional copy of *orf2* into chromosomal DNA.

ΦC31 is a broad-host-range temperate streptomycete phage. ΦC31 derivatives can integrate into many different *Streptomyces* spp., including *S. clavuligerus*. ΦC31 vectors

containing an *oriT* element have shown a consistently high transformation efficiency (1.5×10^5 to 3.8×10^6 in *S. ambofaciens*) as well as a unique integrative site (*attB*) on the chromosome. Plasmids or cosmids that integrate site-specifically at the Φ C31 attachment sites give rise to stable exconjugants (10). They can be propagated without detectable loss of plasmid markers, even in the absence of drug selection (10). Interestingly, Φ C31-driven, site-specific recombination is apparently very efficient since it was reported that plasmids containing as much as 8-kb of homologous DNA were found only at the Φ C31 attachment site, with no detectable integration by homologous recombination (5). This discovery is very important for our study because the constructed recombinant plasmid for *orf2* will contain both a homologous DNA fragment and site-specific integration elements. The preference for site-specific integration will generate strains containing an additional copy of *orf2* inserted site-specifically on chromosomal DNA.

pSET152 is a bifunctional integration plasmid derived from Φ C31 (10). The PCR product of *orf2* and its native promoter sequence was inserted into the *EcoRV* site of pSET152 to give *orf2* integration vector pSET152/pro-*orf2* (Figure 3). This plasmid was introduced into both wild-type and an *orf2* disruption mutant of *S. clavuligerus* (1) (11). The chromosomal DNA isolated from one apramycin resistant transformant was digested with *EcoRI-HindIII* and then hybridized with an *orf2* probe. As shown in Figure 4 (lanes 3 and 4), 12-kb and 1.8-kb hybridization bands indicate that there are two copies of *orf2* in the chromosome, one is the wild-type copy and the other one is the integrated copy cloned on pSET152/pro-*orf2*. Only the 12-kb band was observed in DNA isolated from *S. clavuligerus* transformed with pSET152 indicating the vector has site-specifically integrated into the chromosome.

The integrated copy of *orf2* could restore clavulanic acid production in the *orf2* disruption mutant, indicating that the integrated *orf2* was functional (data not shown). Fermentation in SA+ medium showed that the recombinant strain produced about 66% more clavulanic acid than wild-type strain when the growth rate was identically maintained (Figure 5).

Example 4. Expression of the additional *orf2* gene with the *ermE promoter in *S. clavuligerus*.**

We also used the constitutive and strong *ermE** promoter (4) to investigate the effect of a different promoter on clavulanic acid production in *S. clavuligerus*. *orf2* was excised from its *E. coli* overexpression vector pET24a/*orf2*-*orf3* along with the ribosome binding sequence and placed downstream of the *ermE** promoter in pIJ4070. To obtain maximal expression, *orf2* was inserted into two different sites (*Xba*I and *Hind*III) downstream of *ermE** promoter to generate a 49-bp and a 70-bp region between the -10 sequence and translation initiation codon. A DNA fragment containing the *ermE**(*Xba*I)-*orf2* or *ermE**(*Hind*III)-*orf2* cassette was cloned into pSET152 to generate two integration vectors, pSET152/*ermE*(*Xba*I)-*orf2* and pSET152/*ermE*(*Hind*III)-*orf2* (Figures 6 and 7). The recombinant derivatives were introduced into both the *orf2* disruption mutant, and wild-type of *S. clavuligerus* where integration was successfully observed..

Chromosomal DNA isolated from *S. clavuligerus* transformed with pSET152 or pSET152/*ermE*(*Xba*I)-*orf2* was digested with *Eco*RI, while chromosomal DNA isolated from *S. clavuligerus* transformed with pSET152 or pSET152/*ermE*(*Hind*III)-*orf2* was digested with *Eco*RI-*Hind*III. Southern hybridization with an *orf2* probe showed that strains transformed with recombinant derivatives gave two positive bands (12 kb and 2.2 kb), which correspond to the wild-type copy and the integrated copy of *orf2* on the chromosome, respectively, whereas, strains transformed with pSET152 gave only a 12-kb positive band corresponding to the wild-type *orf2* from the clavulanic acid gene cluster (Figure 4, lanes 1 and 5).

To examine the effect of the *ermE** promoter on the expression of the additional copy of *orf2* and on clavulanic acid production, production levels of antibiotic in SA+ liquid cultures of the wild-type and recombinant strains were determined. Fermentation in shake flasks showed that the *orf2* disruption mutant could be complemented by the integrated *orf2*, indicating the expression of *orf2* under the transcriptional control of *ermE** promoter (data not shown). Wild-type *S. clavuligerus* harboring pSET152 gave the same level of clavulanic acid production as the wild-type strain, indicating that the integration of pSET152 has no negative effect on clavulanic acid production (data not shown). Shake flask fermentation of

both *S. clavuligerus* (pSET152/ermE(XbaI)-orf2) and *S. clavuligerus* (pSET152/ermE(HindIII)-orf2) gave increased yield of clavulanic acid, but the former produces about 34% greater antibiotic and the later gives about 68% more (Figure 8), indicating that the larger separation between the initiation codon and the *ermE** promoter is more efficient for *orf2* expression.

Example 5. Stability of integrative vectors pSET152/pro-*orf2*, pSET152/ermE(XbaI)-*orf2* and pSET152/ermE(HindIII)-*orf2* in *S. clavuligerus*

The stability of all pSET152-derived vectors was tested in their *S. clavuligerus* transformants. The apramycin resistant colonies on the primary plates were transferred onto slants absent selective pressure. After growing for 5 days, a seed medium was inoculated and grown for 72 h and followed by 120 h or 144 h fermentation, both also carried out without selective pressure. Total DNA was isolated from 120 h or 144 h fermentation cultures and transformed *E. coli* DH5 α cells. No apramycin resistant colonies were observed on any of the transformation plates, indicating that there are no free replicating plasmids in these strains after three generations of growth without antibiotic selection.

EXPERIMENTAL

Bacterial strains, vectors and growth conditions.

Streptomyces clavuligerus ATCC27064, *Escherichia coli* JM110, *Escherichia coli* DH5 α were obtained from the American Type Culture Collection, Manassas, VA.

S. clavuligerus was maintained on SP medium (per liter): yeast extract, 10 g; glycerol, 10 g; Bacto-agar, 20 g; pH 6.8. Seed medium consisting of tryptic soy broth (Difco; Detroit, MI) was inoculated with spores of *S. clavuligerus* and grown at 28 °C on a rotary shaker (300 rpm) for 72 h. For clavulanic acid production mycelia from the seed cultures were inoculated into SA medium (13) plus 1% arginine and 1% glycerol at 5%, and this culture was grown under the same conditions as the seed culture. *Escherichia coli* strains were grown in either Luria broth, or TB broth as liquid medium or agar plates (17). The cloning vectors pIJ486, pKC1139 and pSET152 were provided by C. R. Hutchinson (University of Wisconsin,

Madison, WI). The cloning vector pT7Blue-3 and the expression vector pET24a were purchased from Novagen (Madison, WI). The expression vector pIJ4070 was kindly provided by M. J. Bibb (The Johns Innes Institute, Norwich, UK). pL8, a genomic library clone containing clavulanic acid gene cluster (11), is maintained by the Dept. of Chemistry, The Johns Hopkins University (Baltimore, MD).

E. coli and *Streptomyces* plasmid DNA was isolated by standard methods (8) (17) and purified using the Genieprep DNA Isolation Kit (Ambion Inc.; Austin, TX). Genomic DNA from *S. clavuligerus* (ATCC 27064) and disruption mutants were isolated as described by Hopwood (8) and purified with the QIAamp Tissue Kit (Qiagen; Chatsworth, CA).

Transformation of *E. coli* strains was preformed by standard procedures (17).

PCR amplification of *orf2* and its upstream region:

orf2 along with its 500-bp upstream regulatory region was amplified by PCR. Two primers (P5-1-2: AAGCTTATGGGGCCAGGCTATGCG [SEQ ID NO:1] and P3-2-2: GGATTCTCAGATGCTCAGGGCGC [SEQ ID NO:2]) were synthesized. The PCR reaction was carried in a 100 µl system containing 0.5 µg pL8 DNA, 0.2 nM of each primer, 0.2 mM dNTP, 10 µl DMSO and 1 × *Pfu* buffer. After heating for 5 min, 1 µl of *Pfu* DNA polymerase (2 U) (Stratagene, La Jolla, CA) was added. The PCR reaction was carried for 30 cycles, the conditions for the first 5 cycles were: 94 °C, 1 min; 55 °C, 1 min and 30 sec.; 72 °C, 1 min and 30 sec., then the annealing temperature was raised to 58 °C and 25 cycles were performed. In the last cycle, the elongation was carried out for 10 min to ensure the reaction was complete.

Transformation of *S. clavuligerus*.

The conditions for protoplast formation, regeneration, and DNA transformation were modified from the methods of Dominguez, Illing, and Malmberg (7) (9) (12). About 10⁹ spores of wild-type or mutant *S. clavuligerus* were inoculated into 50 ml of TSB broth (14) in a 250-ml flask containing glass beads and grown at 26 °C with rotary shaking for 60 h. Mycelia were harvested by centrifugation, washed twice with 10.3% sucrose and once with P buffer [Tris-HCl, 0.31% (pH 8.0); CaCl₂ 2H₂O 0.368%; MgCl₂ 6H₂O 0.204%; sucrose

10%; glucose 1%]. The pellet was resuspended in P buffer containing 2 mg/ml lysozyme to the final volume of 10 ml and incubated at 30 °C for 25 min. The protoplast/mycelia mixture was filtered through a sterile cotton plug. The protoplasts were collected by centrifugation at 1000 × g for 10 min at 4 °C, washed three times with ice-cold P buffer and diluted to the
5 final concentration of approximately 10⁹/ml. Before DNA transformation, about 10⁸ protoplasts were preheated in a 45 °C water bath for 10 min to inactivate the *S. clavuligerus* restriction system (2). The heat-treated protoplasts were transformed with 2 µg DNA and 500 µl of 25% (wt/vol) polyethylene glycol 1000 (NBS Biologicals, Hatfield, UK) solution was added immediately (8). After incubation at room temperature for 1 min, the transformed
10 protoplasts were diluted with 2.5 ml ice-cold P buffer, collected by centrifugation, and resuspended in 1 ml P buffer. Each pre-dried R₂YEG regeneration plate (12) was plated with 100 µl transformed protoplasts and incubated at 26 °C. The plates were overlaid with 1.5 ml thiostrepton solution at the final concentration of 5 µg/ml or apramycin at 10 µg/ml.

Fermentation and analysis of clavulanic acid:

15 50 ml of TSB seed medium supplemented with glass beads was inoculated with either spores stock or from slants. 100 µg/ml apramycin was added when a strain containing a replicating plasmid was grown, while no antibiotic was added for growth of strains harboring an integrated plasmid. The seed culture was grown at 26 °C for 72 h at 300 rpm shaking. 0.5 ml seed culture was transferred to 50 ml SA+ fermentation medium in a 250 ml
20 flask and incubated at 26 °C shaken as above for 120 or 144 h. 1 ml of culture was taken at 24, 48, 72, 96, 120 and 144 h and centrifuged at 14000 rpm for 5 min.

Clavulanic acid was determined by the β-lactamase inhibition assay with *K. pneumoniae* subsp. *pneumoniae* and benzylpenicillin (15). Clavulanic acid was also detected by reaction with imidazole (6). Filtered fermentation supernatant was reacted with 0.25
25 equiv. vol. of 3 M imidazole reagent (pH 6.8) at 40°C for 20 min. The product of imidazole reaction showed a maximum absorbance at 312 nm (6) (16).

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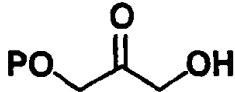
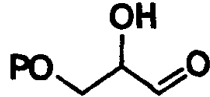
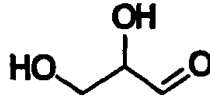
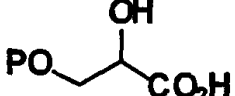
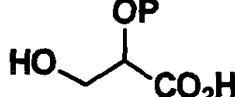
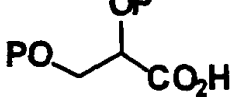
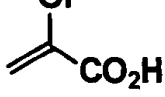
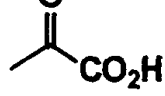
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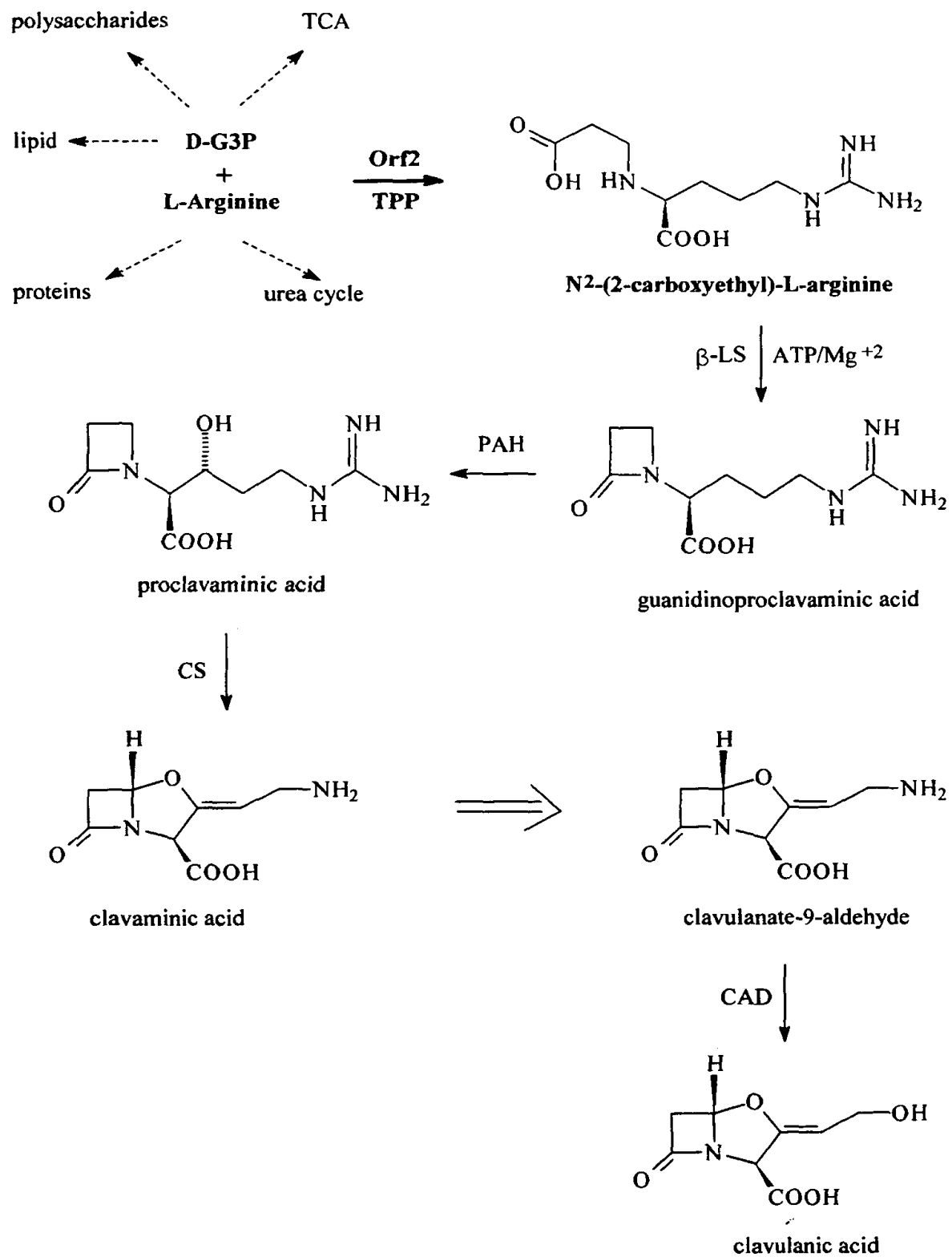
30 While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims. Accordingly, the present invention should not be

limited to the embodiments as described above, but should further include all modifications and equivalents thereof within the spirit and scope of the description provided herein.

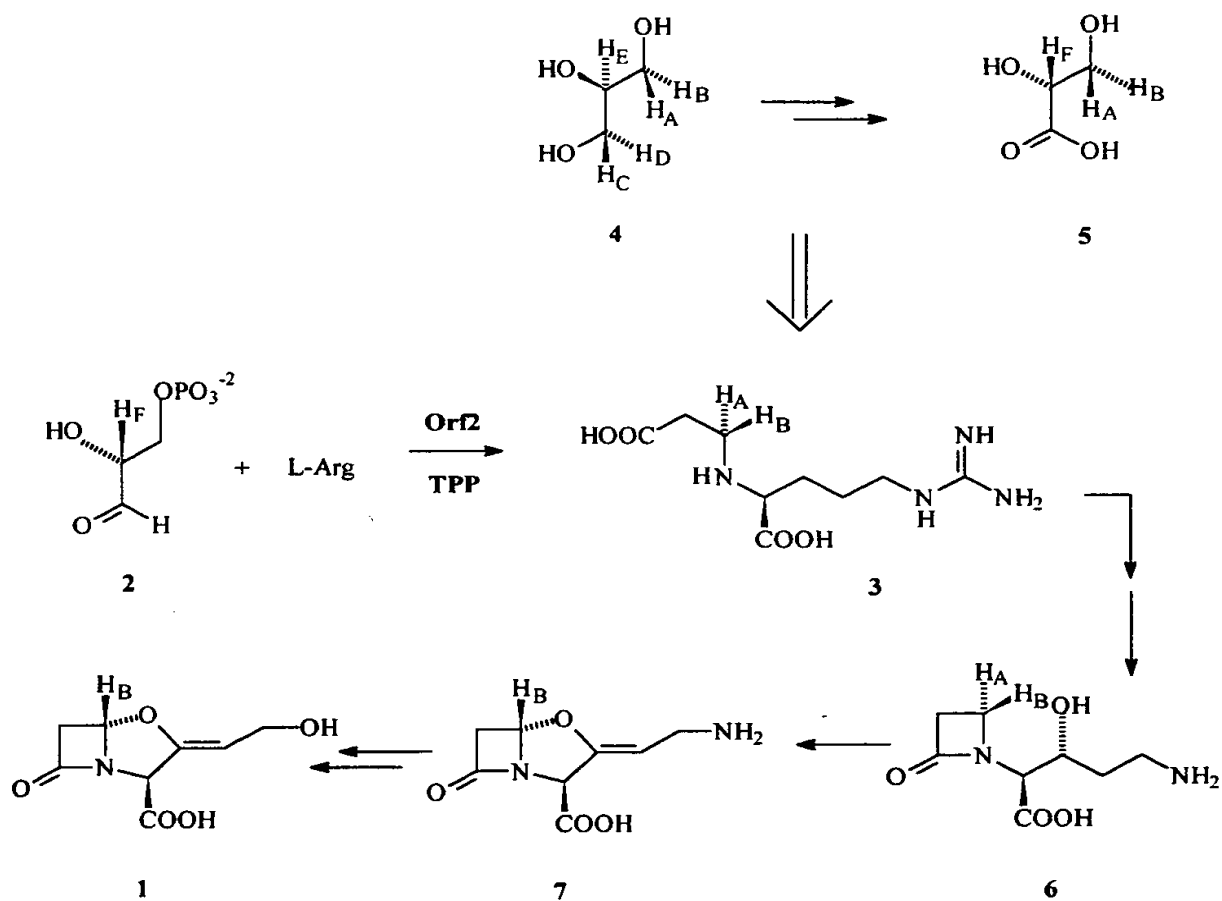
Table 1. Screening of potential C₃-carbohydrates with either a cell-free extract (CFE) of *E. coli* B834(DE3) overexpressing *orf2*, or dialyzed 30% ammonium sulfate pellet containing >95% CEA synthase.

structure	abbrev.	conc. (mM)	% conversion	
			CFE	30% (NH ₄)SO ₂ pellet
	DHAP	30	9.7	29.8
	D,L-G3P	30	3.1	3.9
		60	-	6.1
	D,L-GA	30	3.8	2.4
	3-PGA	30	3.5	0.5
	2-PGA	30	4.6	1.2
	2,3-DPGA	30	3.4	0.3
	PEP	30	2.8	2.6
	PA	30	3.4	1.1

Scheme 1.



Scheme 2.



We claim:

- 1 1. A method for increasing the production of clavulanic acid in a host comprising the
2 step of:
3 increasing the level of N²-(2-carboxyethyl)arginine synthase in said host, wherein
4 said N²-(2-carboxyethyl)arginine synthase catalyzes the condensation of L-arginine and D-
5 glyceraldehyde-3-phosphate, resulting in increased production of clavulanic acid.
- 1 2. The method of claim 1 wherein said step of increasing is performed by gene dosing.
- 1 3. The method of Claim 2 wherein said increasing step is performed by providing said
2 host with DNA encoding said N²-(2-carboxyethyl)arginine synthase.
- 1 4. The method of claim 3 wherein said DNA is in a plasmid.
- 1 5. The method of claim 4 wherein said plasmid is a replicating plasmid.
- 1 6. The method of claim 5 wherein said replicating plasmid is pKC1139/pro-orf2-ter.
- 1 7. The method of claim 3 further comprising integrating said DNA into the
2 chromosome of said host.
- 1 8. The method of claim 7 wherein said DNA is stably integrated via an integrative
2 vector selected from the group consisting of pSET152/pro-orf2, pSET152/ermE(XbaI)-orf2
3 and pSET152/ermE(HindIII)-orf2.

1 9. The method of claim 3 wherein expression of said DNA is under the control of a
2 constitutive promoter.

1 10. The method of claim 3 wherein said constitutive promoter is *ermE**.

1 11. The method of claim 1 wherein said increasing step is performed by adjusting
2 fermentation conditions and/or providing additives which effect the optimization of
3 N²-(2-carboxyethyl)arginine synthase activity, wherein said optimization results in an
4 increase in the production of clavulanic acid

1 12. The method of claim 1 wherein said host is *Streptomyces clavuligerus*.

1 13. A method for increasing the production of clavulanic acid in a host comprising the
2 step of:

3 increasing the availability of precursors for reaction by N²-(2-carboxyethyl)arginine
4 synthase, wherein said step of increasing results in an increase in the production of
5 clavulanic acid.

1 14. The method of claim 13 wherein said precursors are L-arginine and D-
2 glyceraldehyde-3-phosphate.

1 15. The method of claim 13 wherein said host is *Streptomyces clavuligerus*.

1 16. The method of claim 13 wherein said increasing step is achieved by adjusting
2 fermentation conditions and/or providing additives which optimize
3 N²-(2-carboxyethyl)arginine synthase activity.

1 17. A method for increasing the production of N²-(2-carboxyethyl)arginine in a host
2 cell, comprising,

3 enhancing a rate of condensation of L-arginine and D-glyceraldehyde-3-phosphate in
4 said host cell, wherein said step of enhancing results in an increase in the production of
5 N²-(2-carboxyethyl)arginine in said host cell.

1 18. The method of claim 17 wherein said condensation of L-arginine and D-
2 glyceraldehyde-3-phosphate is catalyzed by the enzyme N²-(2-carboxyethyl)arginine
3 synthase.

1 19. The method of claim 17 wherein said step of enhancing is carried out by increasing
2 the copy number of a gene encoding N²-(2-carboxyethyl)arginine synthase.

1 20. The method of claim 17 wherein said step of enhancing is carried out by adjusting
2 fermentation conditions and/or providing additives which optimize
3 N²-(2-carboxyethyl) arginine synthase activity.

1 21. A method for preparing an composition having N²-(2-carboxyethyl)arginine
2 synthase activity, comprising the steps of
3 growing a culture of a host cell capable of synthesizing N²-(2-carboxyethyl)arginine
4 synthase,
5 harvesting and sonicating said culture,
6 removing cellular debris to produce a cellular supernatant,
7 fractionating said supernatant with ammonium sulfate to form a precipitated protein
8 pellet,
9 resuspending said precipitated protein pellet to form a protein solution, and,
10 chromatographing said protein solution by affinity chromatography to isolate a

11 thiaminepyrophosphate-dependent enzyme having N²-(2-carboxyethyl)arginine synthase
12 activity.

1 22. The method of claim 21 wherein said affinity chromatography is carried out with
2 an L-arginine agarose affinity column.

1 23. The method of claim 21 wherein said host is *Streptomyces clavuligerus*.

1 24. The method of claim 21 wherein said step of fractionating is carried out with 30%
2 ammonium sulfate.

1 25. An assay for identifying substrates of the enzyme N²-(2-carboxyethyl)arginine
2 synthase, comprising the steps of

3 incubating a putative substrate with the enzyme N²-(2-carboxyethyl)arginine
4 synthase, thiaminepyrophosphate, and one known substrate of N²-(2-carboxyethyl)arginine
5 synthase, and

6 detecting the presence or absence of a condensation product of the putative substrate
7 and the known substrate, wherein the presence of a condensation product is a positive result.

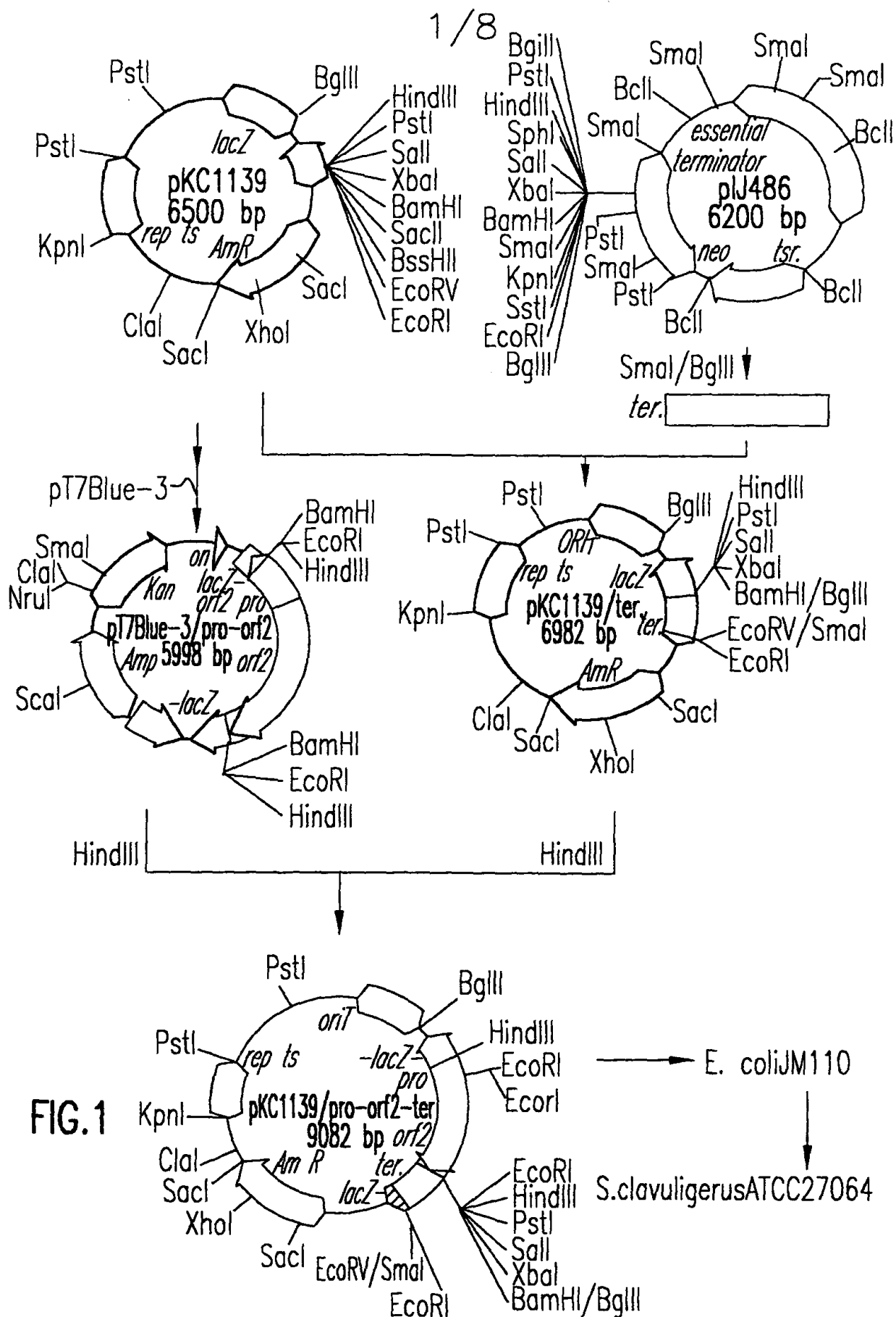
1 26. The assay of claim 25 wherein said known substrate is L-arginine.

1 27. The assay of claim 25 wherein said known substrate is D-glyceraldehyde-3-
2 phosphate.

1 28. A host cell stably transformed with *orf2*.

1 29. The host cell of claim 28, wherein said host cell is *Streptomyces clavuligerus*.

30. A condensation product of two substrates condensed by
N²-(2-carboxyethyl)arginine synthase.



2/8

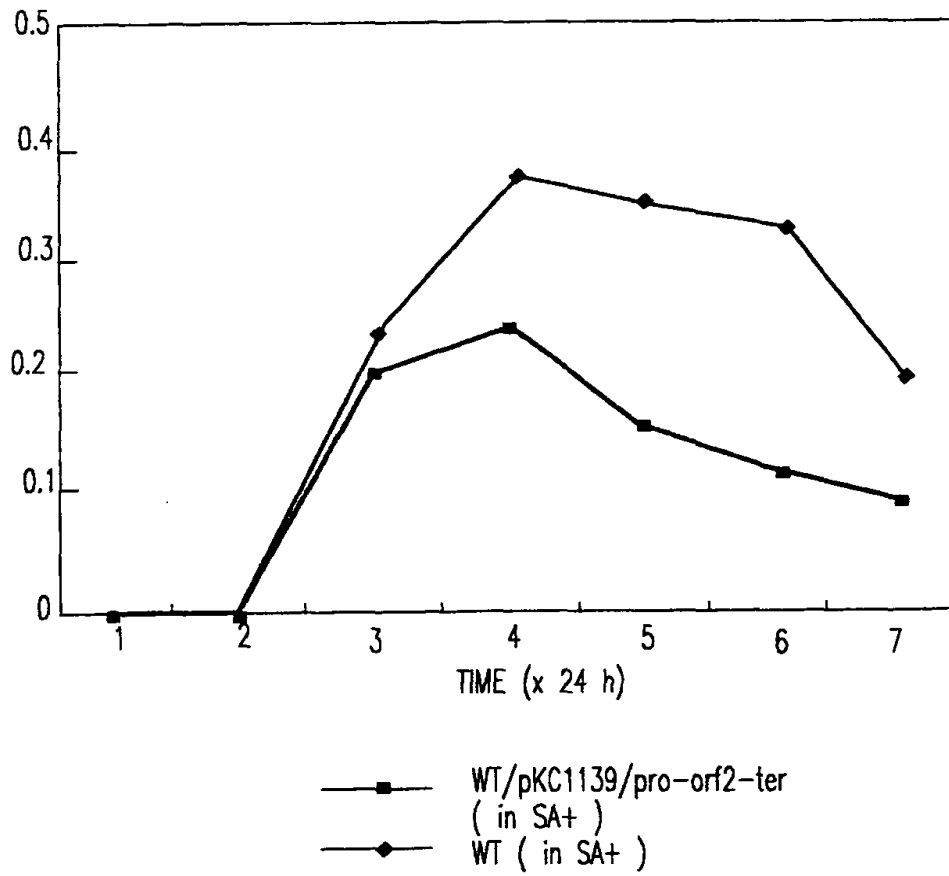


FIG.2

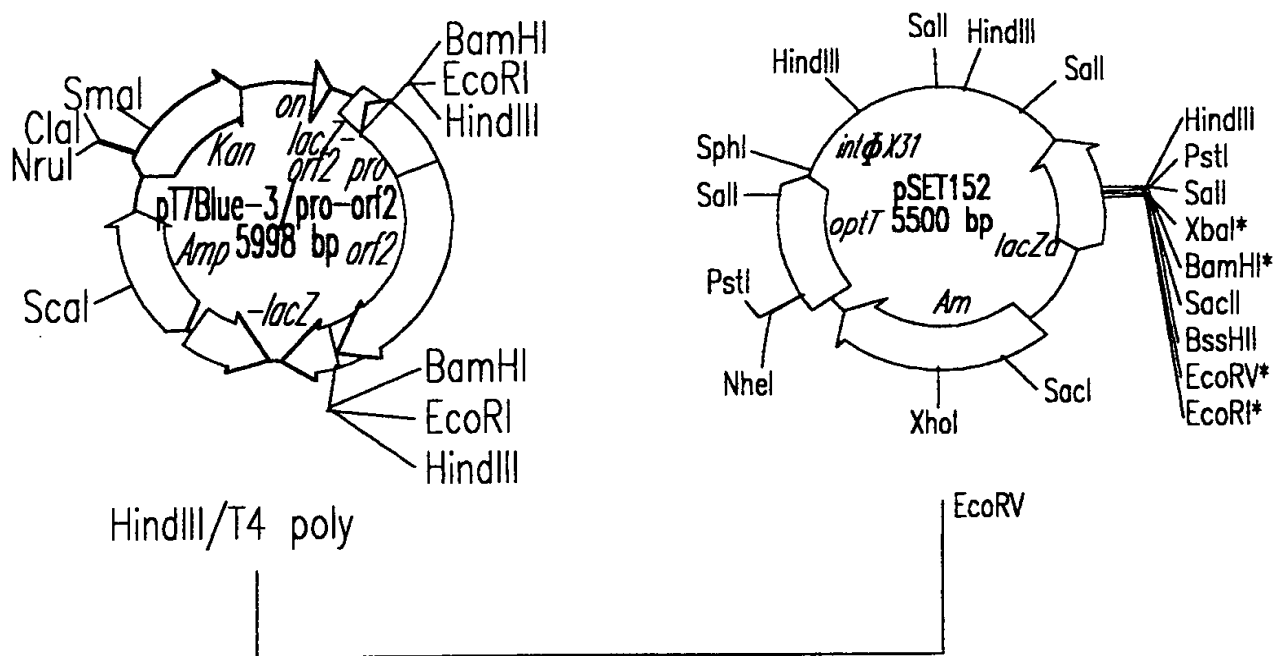
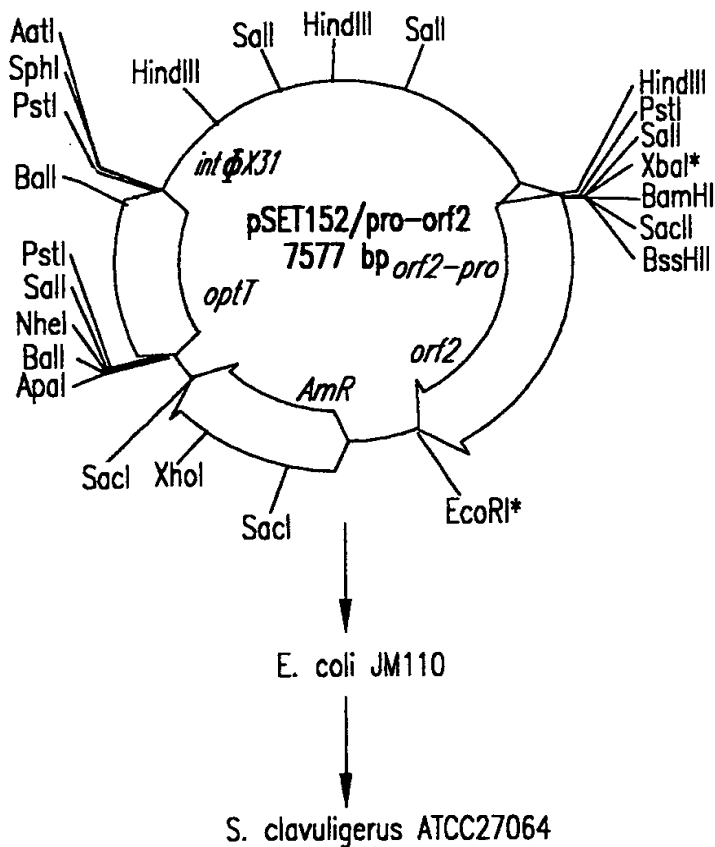


FIG.3



4/8



FIG.4

5/8

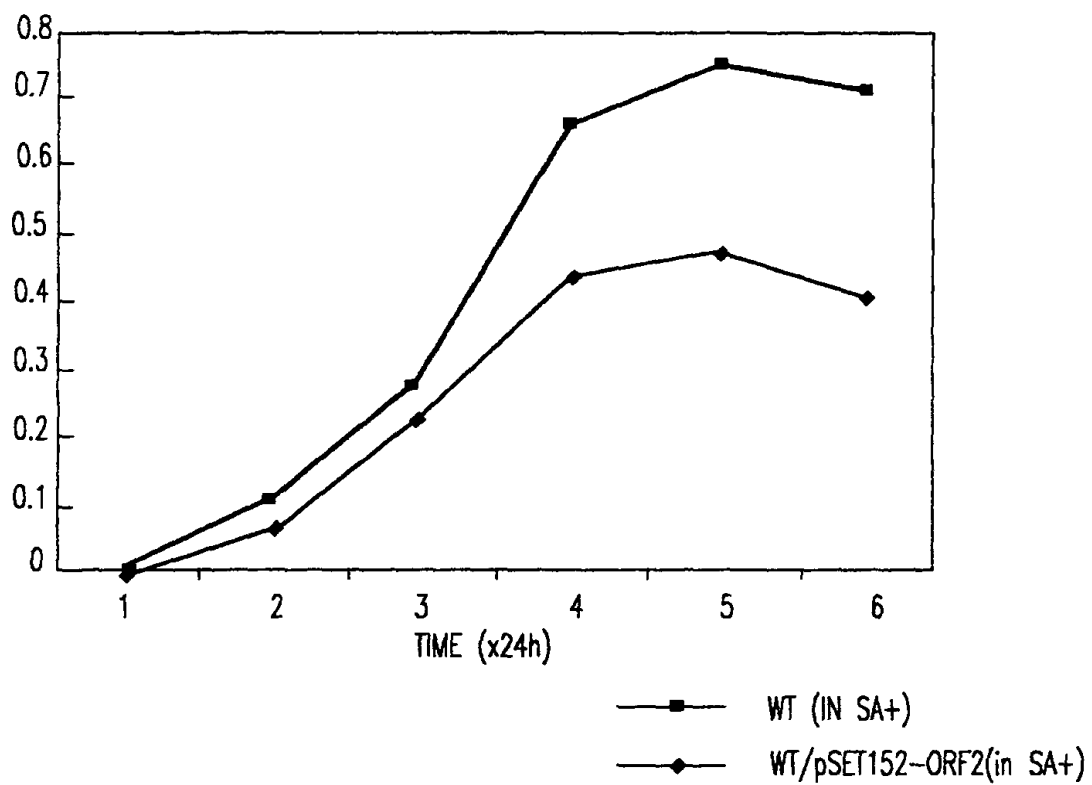


FIG.5

6/8

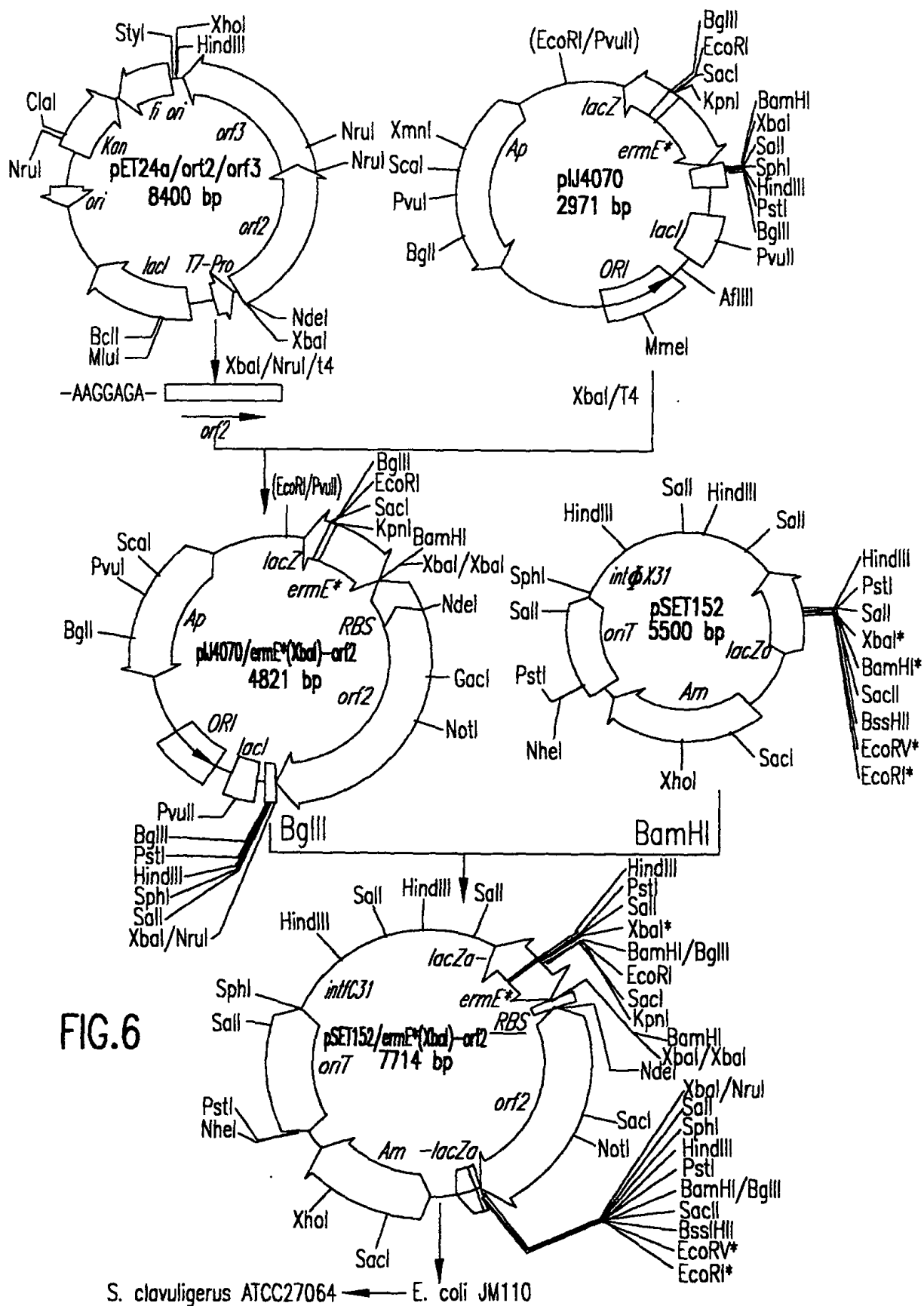


FIG.6

7/8

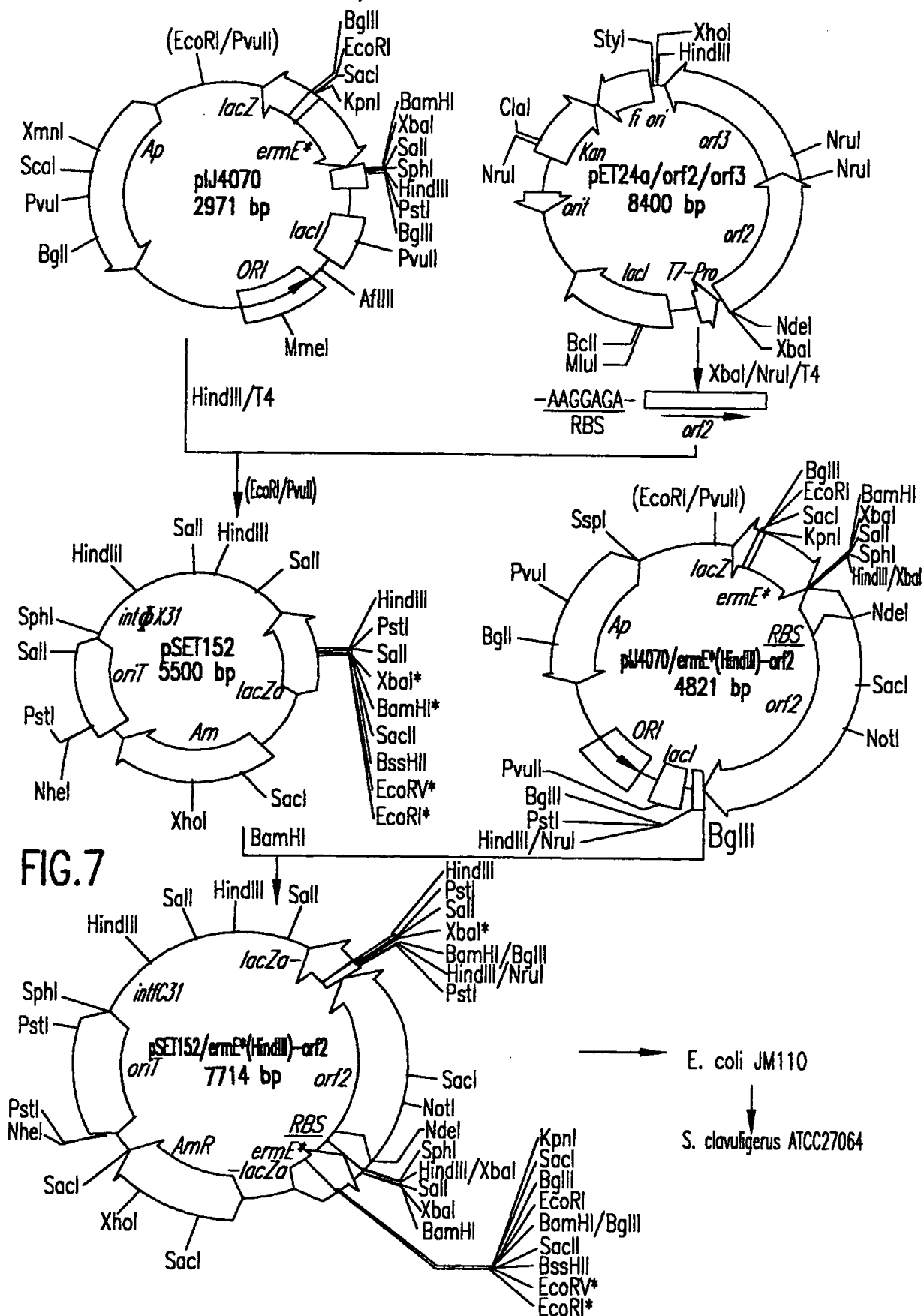


FIG.7

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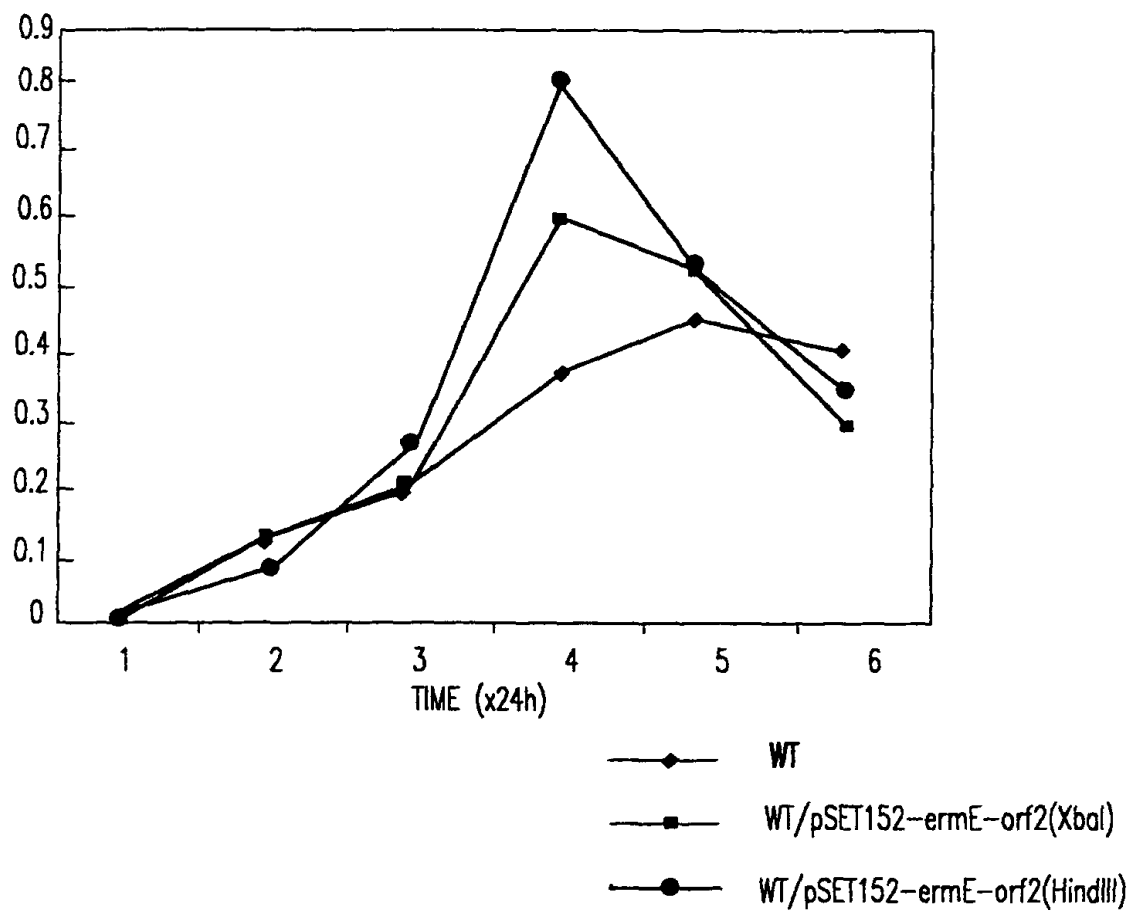


FIG.8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/25186**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : Please See Extra Sheet.

US CL : 536/23.1; 435/ 252.3, 320.1, 326, 471

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/ 252.3, 320.1, 326, 471

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS, WEST**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,869,299 A (BAGGALEY et al) 09 February 1999 (09.02.99), see entire document.	1-30
Y	Database Caplus on STN, No. 583939, KHALEELI, N. et al. 'Origin of the beta-lactam carbons in clavulanic acid from an unusual thiamine pyrophosphate-mediated reaction,' abstract, J. Am. Chem. Soc, 1999, 121(39), 9223-9224.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	-T- later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	-X- document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	-Y- document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	-A- document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 DECEMBER 2000

Date of mailing of the international search report

16 JAN 2001

Name and mailing address of the ISA/US
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Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PETER PARAS JR

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/25186

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C12N 1/20, 15/00, 15/06, 15/09, 15/16, 15/63, 15/70, 15/74; C07H/21/02, 21/04

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: MICHAEL E. WHITHAM
MCGUIREWOODS, LLP
1750 TYSONS BLVD
SUITE 1800
MCLEAN, VA 22102

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing
(day/month/year)

26 MAR 2002

Applicant's or agent's file reference

03940005TA

IMPORTANT NOTIFICATION

International application No.

PCT/US00/25186

International filing date (day/month/year)

15 SEPTEMBER 2000

Priority Date (day/month/year)

16 SEPTEMBER 1999

Applicant

THE JOHNS HOPKINS UNIVERSITY

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB-301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 03940005TA	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/25186	International filing date (day/month/year) 15 SEPTEMBER 2000	Priority date (day/month/year) 16 SEPTEMBER 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE JOHNS HOPKINS UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.	
2. This REPORT consists of a total of <u>6</u> sheets.	
<input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).	
These annexes consist of a total of <u>0</u> sheets.	
3. This report contains indications relating to the following items:	
I <input checked="" type="checkbox"/> Basis of the report	
II <input type="checkbox"/> Priority	
III <input type="checkbox"/> Non-establishment of report with regard to novelty, inventive step or industrial applicability	
IV <input type="checkbox"/> Lack of unity of invention	
V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability, citations and explanations supporting such statement	
VI <input type="checkbox"/> Certain documents cited	<input type="checkbox"/> contained in the international application
VII <input checked="" type="checkbox"/> Certain defects in the international application	<input type="checkbox"/> filed together with the international application
VIII <input checked="" type="checkbox"/> Certain observations on the international application	<input type="checkbox"/> furnished subsequently to the international application
	<input type="checkbox"/> furnished with the international application as the basis for the report
	<input type="checkbox"/> This report is based on the international application as the basis for the report
	<input type="checkbox"/> The international application has been furnished.

Date of submission of the demand 21 DECEMBER 2000	Date of completion of this report 06 MARCH 2002	<input checked="" type="checkbox"/> the description pages <input checked="" type="checkbox"/> the claims, pages <input checked="" type="checkbox"/> the drawings, sheets
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <i>Peter Paras, Jr.</i> PETER PARAS, JR.	
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196	

I. Basis of the report

1. With regard to the elements of the international application:*

- ☒ the international application as originally filed
- ☒ the description:
pages 1-27, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the claims:
pages 28-32, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the drawings:
pages 1-8, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in printed form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

- ☒ the description, pages NONE
- ☒ the claims, Nos. NONE
- ☒ the drawings, sheets/fig NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

**Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/25186

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims 1-29	YES
	Claims 30	NO
Inventive Step (IS)	Claims 1-29	YES
	Claims 30	NO
Industrial Applicability (IA)	Claims 1-30	YES
	Claims NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-29 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest methods for increasing the production of clavulanic acid in a host.

Claim 30 lacks novelty under PCT Article 33(2) as being anticipated by Baggaley et al (US 5,869,299).

Baggaley et al teach a condensation product of two substrates condensed by N^ε-(2-carboxyethyl) arginine synthase. The teachings of Baggaley anticipate the claimed invention.

----- NEW CITATIONS -----

KHALEELI et al. Origin of the B-lactam carbons in clavulanic acid from an unusual thiamine pyrophosphate-mediated reaction. J. Am. Chem. Soc. 1999, Vol. 121, pages 9223-9224, see entire document.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/25186

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 21 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: the term "capable of" is indefinite. It is unclear from the claim as written if the host cell actually produces N^ε-(2-carboxyethyl) arginine synthase.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-22 and 24-28 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the following paragraph. The description is enabling for a method of increasing the amount of clavulanic acid in *Streptomyces clavuligerus* comprising introducing a nucleic acid sequence encoding N²-(2-carboxyethyl) arginine synthase into *Streptomyces clavuligerus*, wherein the amount of clavulanic acid is increased. The description is not enabling for other methods of increasing the amount of clavulanic acid as claimed. The description is not enabling for use of the methods as claimed in cells other than *Streptomyces clavuligerus*. It is unpredictable if other cells (including eukaryotic cells) can produce clavulanic acid. Khaleedi et al teach that clavulanic acid is produced by bacteria, particularly *Streptomyces clavuligerus*.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/25186

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C12N 1/20, 15/00, 15/06, 15/09, 15/16, 15/63, 15/70, 15/74; C07H/21/02, 21/04 and US Cl.: 536/23.1; 435/252.3, 320.1, 326, 471